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Neural Crest Cells and Malignant Triton Tumor Lines

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13. ABSTRACT (Maximum 200 Words) Our purpose is to examine the role of the Nf1 gene product, neurofibromin, in modulating the migratory and invasive properties of neural crest cells (NCC) and neural crest-derived sarcoma cells. As a negative regulator of Ras signaling, neurofibromin may influence the responses of NC-derived cells to growth factors and extracellular matrix (ECM) molecules that affect motility. We use embryonic NCC and NC-derived sarcoma lines isolated from cisNf1;p53 mice to compare integrin ECM receptor expression patterns, ECM adhesion preferences, migration on ECM substrata, invasion through ECM barriers, and dispersal along NCC pathways in vivo for wild-type and neurofibromin-deficient cells. In the past year, we have completed studies on the invasiveness of branchial arch mesenchymal cells and trigeminal neural crest cells isolated from Nf-/-, +/-, and +/+ mouse embryos. Consistent with published results for neurofibromin-deficient astrocytes, mast cells, and Schwann cells, our data indicate that Nf1 mutant cranial neural crest cell populations are more invasive through laminin and fibronectin matrices. We have also examined the effects of fibroblast growth factor, a chemoattractant molecule for early-migrating mesencephalic neural crest cells, and shown that responsiveness to this molecule declines with age in utero. Finally, we have begun to perturb signaling intermediates downstream of neurofibromin and Ras, in an attempt to inhibit or alter the invasiveness of neurofibromin-deficient cells.				
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INTRODUCTION

The neural crest of vertebrate embryos is a migratory population of stem cells that give rise to sensory and autonomic neurons, Schwann cells, melanocytes, and smooth muscle cells in the outflow tract of the heart (Weston, 1991). Neurofibromatosis type 1 (NF1) is in large part a disorder of neural crest-derivatives, and the neurofibromin protein regulates the responses of neural crest-derived neurons, Schwann cells, and melanocytes to a variety of environmental cues. Mice harboring targeted null mutations in the *Nf1* and *Trp53* tumor suppressor genes in the cis configuration spontaneously develop malignant soft tissue sarcomas, including malignant peripheral nerve sheath tumors and malignant Triton tumors (Cichowski et al., 1999; Vogel et al., 1999). The objectives of the proposed research are to 1) analyze the effects of *Nf1* gene dosage on the migratory and invasive properties of primary neural crest cells isolated from E9-E11.5 mouse embryos, and 2) to characterize the contributions of TGF β and Ras signaling to the invasive and motile properties of neural crest-derived cis*Nf1*;p53 sarcoma cells, and of primary neural crest cells isolated from mouse embryos.

BODY

Task 1: Compare neural crest cell (NCC) dispersal in wild-type and *Nf1*^{-/-} mouse embryos

Our analyses of cranial neural crest migration in *Nf1*^{-/-} mouse embryos revealed no obvious differences from that in wild-type embryos, with respect to: 1) localization in branchial arches, 2) localization in trigeminal ganglion, and 3) initial axon outgrowth from trigeminal ganglion neurons. In contrast to the enlarged dorsal root sensory ganglia in the trunks of *Nf1*^{-/-} mouse embryos (Lakkis et al., 1999), we find that the trigeminal ganglia of neurofibromin-deficient embryos are smaller than those of stage-matched *Nf1*^{+/+} and +/- littermates (C. Geyer and K.S. Vogel, unpublished results). However, trigeminal ganglia develop in the appropriate location in *Nf1*^{-/-} mouse embryos, and appear to initiate axon outgrowth, and to undergo normal pathfinding to peripheral and central targets (R. Erzurumlu and K.S. Vogel, unpublished results).

Task 2: Compare expression of integrin ECM receptors and motile characteristics for wild-type and *Nf1*^{-/-} mouse NCC

During the past year, we obtained sufficient data on the invasiveness of branchial arch mesenchymal cells and trigeminal neural crest cells, isolated from neurofibromin-deficient embryos, to prepare a manuscript (to be submitted to either *Experimental Cell Research* or *Developmental Dynamics* in November 2003). To present the data for this report, I will use the Results sections and figures from this manuscript. A summary of the significant conclusions and future directions will be presented in the "so what" section.

Branchial arch mesenchymal cells are more invasive than embryonic trunk fibroblasts.

To compare the invasiveness of cranial neural crest cells and trunk fibroblasts, we isolated mandibular arch mesenchymal cells, or body wall and limb fibroblasts, from the E11.5 progeny of *Nf1*^{+/-} matings. Because both fibronectin and laminin play pivotal roles in the migration and localization of neural crest cells (Desban and Duband, 1997; Testaz et al., 1999), we measured invasiveness separately through matrices composed of these proteins in transwell assays. **Figure 1A** shows that for any given *Nf1* genotype, mandibular arch mesenchymal cells are much more invasive than are fibroblasts isolated from the body wall and limbs of the embryo. First arch cells isolated from *Nf1*^{-/-} mouse embryos exhibit increased invasiveness through both fibronectin and laminin when compared to cells isolated from *Nf1*^{+/-} and *Nf1*^{+/+} littermates (**Figure 1A** and see below). To compare the invasiveness of another neural crest-derived cell population, from the trigeminal ganglion, with that of soft tissue sarcoma lines, we measured migration through fibronectin and laminin matrices in the transwell assays. **Figure 1B** shows the results for trigeminal neural crest cells isolated from E11.5 *Nf1*^{-/-}, *Nf1*^{+/-}, and *Nf1*^{+/+} embryos, for 4 different

neural crest-derived soft tissue sarcoma lines (Tu8-2, Tu9-6, Tu19-7, Tu26-6, all *Nf1*^{-/-}; *p53*^{-/-}), for an *Nf1*^{+/-}; *Trp53*^{-/-} sarcoma line (Tu24-4), for an *Nf1*^{+/+}; *p53*^{-/-} (Tu39) sarcoma line, and from an *Nf1*^{-/-}; *Trp53*^{-/-} glioma line (Tu96i-4). The 4 neural crest-derived sarcoma lines were isolated from spontaneously arising tumors in *cisNf1*^{+/-}; *Trp53*^{+/-} mice and all exhibited loss-of-heterozygosity at the *Nf1* and *Trp53* loci (Vogel et al., 1999). Tu8-2 has a relatively undifferentiated neural crest phenotype, Tu9-6 expresses smooth muscle markers, Tu19-7 exhibits a predominantly neuronal phenotype, and Tu26-6 expresses high levels of Schwann cell markers (Vogel et al., 1999). In general, cells that lack neurofibromin (*Nf1*^{-/-}) are more invasive than those that are *Nf1*^{+/-} or *Nf1*^{+/+}, regardless of *Trp53* status (**Figure 1B**). Embryonic *Nf1*^{-/-} trigeminal neural crest cells are as invasive through laminin as are the most aggressively motile neural crest-derived sarcoma lines (Tu19-7, Tu26-6). The glioma line Tu96i-4, isolated from a spontaneously-arising tumor in a *cisNf1*^{+/-}; *Trp53*^{+/-} mouse, exhibits the highest level of invasiveness through a fibronectin matrix (**Figure 1B**).

Loss of neurofibromin increases the invasiveness of first branchial arch mesenchymal cells. To determine whether *Nf1* gene dosage could affect invasiveness of cranial neural crest cell populations, we isolated first arch mesenchymal populations from *Nf1*^{+/+}, *Nf1*^{+/-}, and *Nf1*^{-/-} mouse embryos and compared their performance in transwell migration assays. Invasiveness through either fibronectin or laminin matrix was measured in vitro for mandibular arch mesenchymal cells isolated from E10.0-E10.5 embryos, and for both maxillary and mandibular arch mesenchymal cells isolated from E11.0-E11.5 embryos. **Figure 2A** shows that mandibular arch mesenchymal cells isolated from E10 *Nf1*^{-/-} embryos exhibit increased invasiveness through laminin, but not fibronectin, when compared to cells isolated from *Nf1*^{+/-} and *Nf1*^{+/+} littermates. At E11.5, both maxillary and mandibular arch mesenchymal cells isolated from *Nf1*^{-/-} embryos show increased invasiveness through both fibronectin and laminin matrices (**Figure 2B**). In contrast to results obtained with adult mouse astrocytes (Gutmann et al., 2001) and mast cells (Ingram et al., 2000), we observed no significant differences in invasiveness between first arch mesenchymal cells isolated from *Nf1*^{+/-} and *Nf1*^{+/+} embryos at any stage examined.

Loss of neurofibromin alters the proliferative potential of several different cell populations, including Schwann cells (Kim et al., 1997), astrocytes (Gutmann et al., 1999), and sympathetic neuroblasts (Vogel and Parada, 1998). To determine whether the increased invasiveness of *Nf1*^{-/-} branchial arch cells could be attributed in part to differences in proliferation, we compared BrdU incorporation in mesenchymal cell populations from E10 and E11 maxillary and mandibular arches. For the 24-hr BrdU labeling period, values between 8 and 18.5% were obtained for the proportion of cells that entered S-phase in both neurofibromin-deficient and wild-type cultures (**Table 1**). Thus, under the culture conditions of the invasiveness assays, no differences in the percentage of BrdU⁺ cells were observed for first arch mesenchymal cells isolated from neurofibromin-deficient (*Nf1*^{-/-}) and *Nf1*^{+/-} or *Nf1*^{+/+} mouse embryos.

Table 1. BrdU-labeling of branchial arch mesenchymal populations

Source	Genotype	Average BrdU+ (%)	Range (%)
E10 mandibular arch	<i>Nf1</i> ^{+/-}	13.1	10.3-17.6
E10 mandibular arch	<i>Nf1</i> ^{-/-}	13.4	9.6-18.2
E11 maxillary arch	<i>Nf1</i> ^{+/+}	12.3	10.4-15.2
E11 maxillary arch	<i>Nf1</i> ^{-/-}	11.9	10.6-14.0
E11 mandibular arch	<i>Nf1</i> ^{+/+}	11.6	8.6-13.1
E11 mandibular arch	<i>Nf1</i> ^{-/-}	11.6	9.3-13.8

Response of first branchial arch mesenchymal cells to FGF2 as a chemoattractant.

FGF2 acts as a chemoattractant for mouse mesencephalic neural crest cells in transwell invasiveness assays (Kubota and Ito, 2000). To determine whether first arch mesenchymal cells would also respond to FGF2 with increased migration, and to compare the responsiveness of *Nf1*^{-/-}, *+/+*, and *-/-* cells to different concentrations of this factor, we performed transwell invasiveness assays with first arch mesenchymal cells using FGF2 as a chemoattractant. **Figure 3A** shows that FGF2, at 1 ng/ml or 10 ng/ml, has a slight chemoattractive effect on E11.5 *Nf1*^{-/-} maxillary arch mesenchymal cells, when compared to DMEM alone. FGF2 at 10 ng/ml, but not 1 ng/ml, slightly stimulates invasiveness of E11.5 maxillary arch cells isolated from *Nf1*^{+/+} littermates. For mandibular arch mesenchymal cells, reproducible effects of FGF2 on invasiveness were observed only for populations isolated at E10.5 (**Figure 3B**). At 10 ng/ml, FGF2 is slightly chemoattractive for E11 *Nf1*^{-/-} mandibular arch mesenchymal cells; no significant differences were observed for E11 *Nf1*^{+/+} and *+/+* populations. FGF2 has no detectable chemoattractive effect on mandibular arch mesenchymal cells isolated from E11.5 mouse embryos (**Figure 3B**).

Loss of neurofibromin increases the invasiveness of trigeminal ganglion neural crest cells.

Branchial arches consist of cells derived from all three germ layers, including ectodermal and endodermal epithelia, paraxial mesoderm, and neural crest ectoderm. Although we removed all epithelia and most differentiated muscle prior to performing the invasiveness assays, the remaining mesenchymal cell population most likely contained both neural crest and mesodermal cells; moreover, the neural crest cell branchial arch population itself is heterogeneous in developmental potential. Therefore, to measure specifically the invasiveness of a cranial neural crest cell population without contaminating mesodermal cells, we isolated trigeminal ganglion cell populations from mouse embryos between E10.5 and E12.5. In avian embryos, trigeminal ganglion sensory neurons arise from both neural crest and placodal precursors, whereas the support cells are derived from neural crest originating in rhombomere 2 (Krull, 2001). To compare the invasiveness of *Nf1*^{-/-}, *Nf1*^{+/+}, and *Nf1*^{+/+} trigeminal ganglion neural crest cells, we performed transwell assays with fibronectin and laminin matrices. The majority of trigeminal ganglion cells used in these assays, whether isolated from *Nf1*^{+/+} or *Nf1*^{-/-} embryos did not express the neuronal marker NFM. One-third of the cells expressed detectable levels of nestin, and the majority of cells were immunopositive for GAP-43 (data not shown). **Figure 4A** shows that E10.5 trigeminal neural crest cells isolated from *Nf1*^{-/-} embryos exhibited increased invasiveness through laminin, when compared to those isolated from *Nf1*^{+/+} or *Nf1*^{+/+} littermates. *Nf1*^{-/-} trigeminal ganglion cells isolated from E11.5 mouse embryos showed increased invasiveness through both fibronectin and laminin matrices (**Figure 4B**); again, no significant differences were observed between *Nf1*^{+/+} and *Nf1*^{+/+} trigeminal neural crest cells. Both neurons and glial cells in the superior cervical ganglion, part of the sympathetic nervous system, are derived from neural crest cells. To determine if *Nf1* deficiency affected the invasiveness of these autonomic ganglion cells, we compared the performance of *Nf1*^{+/+} and *Nf1*^{-/-} E12.5 SCG cells in the transwell assays. No significant differences in invasiveness through fibronectin or laminin matrix were observed for SCG cells (data not shown).

Pre-treatment of trigeminal ganglion neural crest cells with NGF does not increase invasiveness.

Trigeminal sensory neurons isolated from *Nf1*^{-/-} mouse embryos survive and extend neurites in the absence of nerve growth factor (NGF), whereas those neurons isolated from *Nf1*^{+/+} or *+/+* littermates undergo apoptosis within 48 hours unless this neurotrophin is added to the culture medium (Vogel et al., 1995). We observed that neural crest-derived sarcoma cells that expressed neuronal traits are more invasive, particularly through fibronectin, than are tumor cells that express smooth muscle traits (**Figure 1**). Therefore, to eliminate the possible effects of differential survival and invasiveness of neuronal cells in trigeminal populations isolated from *Nf1*^{-/-} embryos, we maintained trigeminal ganglion cells in the presence or absence of NGF prior to performing invasiveness assays. NGF pre-treatment increases the

proportion of Nf1^{+/-} trigeminal ganglion cells that express the intermediate neurofilament subunit (NFM); in contrast, there is no significant difference in the percentage of NFM⁺ cells for Nf1^{-/-} trigeminal cultures in the presence or absence of NGF. NGF pre-treatment produced no significant differences in invasiveness through laminin for E11.5 trigeminal neural crest cells of any genotype (Figure 5). However, NGF-treated populations of E11.5 trigeminal ganglion cells isolated from Nf1^{+/+} and Nf1^{+/-} embryos exhibited increased invasiveness through fibronectin (Figure 5).

Task 3: Correlate integrin expression, cell adhesion, and responses to TGFβ1 with invasive and metastatic ability in mouse MTT sarcoma lines.

Task 4: Analyze role of Ras signaling in locomotory, invasive, and metastatic properties of mouse MTT sarcoma lines.

Our initial analyses of Ras activation in mouse MTT sarcoma lines this year revealed no significant differences between invasive and non-invasive lines (data not shown). Therefore, we began to focus our attention on signaling pathways downstream of Ras that are known to affect cell motility. Since we could readily control Nf1 gene dosage in populations of embryonic mouse neural crest cells, whereas all of our MTT sarcoma lines derived from cisNf1;Trp53 adults are Nf1^{-/-};Trp53^{-/-}, we have concentrated on these primary cell populations. Phosphatidylinositol-3 kinase (PI3 kinase) can activate Rac or Rho signaling to modulate cell motility in a variety of contexts, and this signaling molecule can be inhibited by treatment with wortmannin or LY294002 (Sachdev et al., 2002). Figure 6 shows that pretreatment with wortmannin (10 μM) or LY294002 (50 μM) reduces the invasiveness of E11.0 branchial arch mesenchymal cells through laminin matrices. We have obtained similar results with trigeminal neural crest cells, and in both cases, Nf1^{-/-} cells appear to be more sensitive to the inhibitory effects of LY294002. Recently, Rangwala and colleagues (2003) have reported that PI3 kinase inhibitors reduce embryonic Schwann cell motility, and that the increased invasiveness of Nf1^{-/-} Schwann cells is mediated by the small GTPase TC21. In the next year, we will examine the effects of activated or dominant negative variants of TC21 and other small GTPases (generously provided by Dr. N. Ratner) on the invasiveness of Nf1^{-/-} and Nf1^{+/+} cranial neural crest cell populations.

KEY RESEARCH ACCOMPLISHMENTS

- Completed comparisons of invasiveness through fibronectin and laminin matrices for branchial arch mesenchymal cells and trigeminal ganglion neural crest cells isolated from Nf1^{-/-}, ^{+/-}, and ^{+/+} mouse embryos.
- Examined roles of NGF and FGF2 in altering cell motility for trigeminal neural crest cells and branchial arch mesenchymal cells isolated from normal and neurofibromin-deficient mouse embryos.
- Prepared manuscript based on above data, to be submitted to *Experimental Cell Research* November 2003.
- Compared Ras activation in several MTT sarcoma cell lines.
- Initiated experiments to inhibit pharmacologically signaling intermediates downstream of Ras and neurofibromin, in branchial arch mesenchymal populations and trigeminal neural crest cells isolated from Nf1^{+/+}, ^{+/-}, and ^{-/-} mouse embryos.
- Established 10 new MTT sarcoma cell lines from cisNf1^{+/-};Trp53^{+/-};lacI⁺ mice, to begin to examine correlation between genomic instability and malignant phenotype (proliferation, invasiveness) in NF1-associated tumors.

REPORTABLE OUTCOMES

Manuscripts

Miller, S.J., Li, H., Rizvi, T.A., Huang, Y., Johansson, G., Bowersock, J., Sidani, A., Vitullo, J., Vogel, K.S., Parysek, L.M., DeClue, J.E., and Ratner, N. (2003) Brain lipid binding protein in axon-Schwann cell interactions and peripheral nerve tumorigenesis. *Mol. Cell. Biol.* 23, 2213-2224

White, C., and Vogel, K.S. Increased invasiveness of neurofibromin-deficient branchial arch mesenchymal and trigeminal ganglion neural crest cells. *To be submitted November 2003*

Abstracts

White, C., and Vogel, K.S. Increased invasiveness of neurofibromin-deficient cranial mesenchymal populations. *Poster presentation at the 2003 NNFF International Consortium for the Molecular Biology of NF1 and NF2*

Garza, R. Deming, B., Walter, C.A., and Vogel, K.S. Mutant frequencies in NF1 tumors and Nf1+/-;Trp53+/- tissues. *Presented by K.S. Vogel at the 2003 NNFF International Consortium for the Molecular Biology of NF1 and NF2*

Employment and Training Opportunities

Christopher White, M.D. Dr. White is a resident in the Department of Surgery at UTHSCSA, and has performed and analyzed some of the invasiveness assays. He is a co-author on the manuscript to be submitted in November 2003.

Rene Garza, Research Assistant. Mr. Garza joined my laboratory in November 2002, and has been instrumental in maintaining and genotyping the mouse colony. He assists with non-survival surgeries in adult mice, and is learning microdissection techniques in mouse embryos. Mr. Garza also assists with in vitro invasiveness assays.

Rose Cantu, Summer Undergraduate Research. Ms. Cantu worked in my laboratory through the UTHSCSA Hispanic Dental Center of Excellence summer research program. She compared spreading behavior on different matrix molecules and invasiveness in response to growth factors for several of the MTT sarcoma cell lines. Ms. Cantu presented her research in poster format in October 2003, as part of the Hispanic Dental Association meeting in Washington DC.

Brenda Deming, Emily Copes, and Jacey Hornecker, Graduate Student Rotation Projects. These three Ph.D. students in our department contributed to isolating new MTT cell lines from cisNf1+/-;Trp53+/-;lacI+ mice, and assisted with genotyping and rodent surgeries.

Funded and Pending Grant Applications

Active

Dates: June 1, 2003- May 31, 2004 Agency: San Antonio Area Foundation Award, (\$11,815) Title: "Mutant Frequency in a Mouse Tumor Model for Neurofibromatosis Type 1" Goals: To compare spontaneous mutant frequency in tumor, brain, and liver tissues isolated from cisNf1+/-;Trp53+/- mice.

Pending

Agency: NIH, (\$1,268,3000) Title: "Genomic Instability in Mouse Models for NF1" Goals: To correlate genomic instability with malignant progression in *cisNf1*^{+/-};*Trp53*^{+/-} tumors, and to characterize genomic instability in the developing nervous system in the context of *Nf1* deficiency.

CONCLUSIONS

Importance, Implications, and "So What" Section

Over the past year, our research has confirmed that neurofibromin modulates motility in a cell type relevant to the manifestations of neurofibromatosis type 1. Specifically, we have used cranial neural crest and mesenchymal cell populations, isolated from early mouse embryos, to demonstrate that neurofibromin-deficient cells are more invasive through extracellular matrices than are cells isolated from normal littermates. Our results are consistent with those obtained for astrocytes (Gutmann et al., 2001), mast cells (Ingram et al., 2000), and Schwann cells (Kim et al., 1997; Rangwala et al., 2003), all of which are potentially affected in human NF1 patients. However, our experiments address the role of neurofibromin in modulating motility for an embryonic cell population that is relatively undifferentiated, and that exhibits highly migratory behavior at the time of isolation. In addition, the experiments with FGF2 indicate that the responsiveness of migratory neural crest cells to chemoattractive molecules, and to other environmental cues, changes during development. For the remainder of the funding period, we plan to identify signaling intermediates that are critical to this altered motility in neurofibromin-deficient neural crest cells, and to challenge the migratory capabilities of these cells before and after treatment with pharmacological inhibitors or dominant negative constructs.

The differences in invasiveness, adhesion to ECM molecules, migration on ECM substrata, and migration in vivo that we have observed between MTT sarcoma lines isolated from *cisNf1*^{+/-};*Trp53*^{+/-} mice indicate that malignant progression and metastatic potential are not dependent solely on Ras activation (through loss of *Nf1*) and loss of p53 function. According to the "mutator phenotype" hypothesis, mutations in genes that control genomic stability occur early in tumorigenesis, and the consequent accumulation of mutations in critical cell cycle, motility, and apoptotic regulatory genes fuels malignant progression. To begin to address this hypothesis in the context of NF1-associated tumors, we are applying both *lacI* mutant frequency measurements and chromosomal analyses to our *cisNf1*^{+/-};*Trp53*^{+/-} mouse tumors, in combination with existing data, obtained under this award, on MTT motility, proliferation, and invasiveness. We believe that we have developed an ideal model in which to examine the consequences of genomic instability for malignant progression in spontaneously-developing tumors that are associated with neurofibromatosis type 1.

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Figure 1A.
Invasiveness of E11.5 Mandibular Arch
Mesenchymal Cells and Trunk Fibroblasts

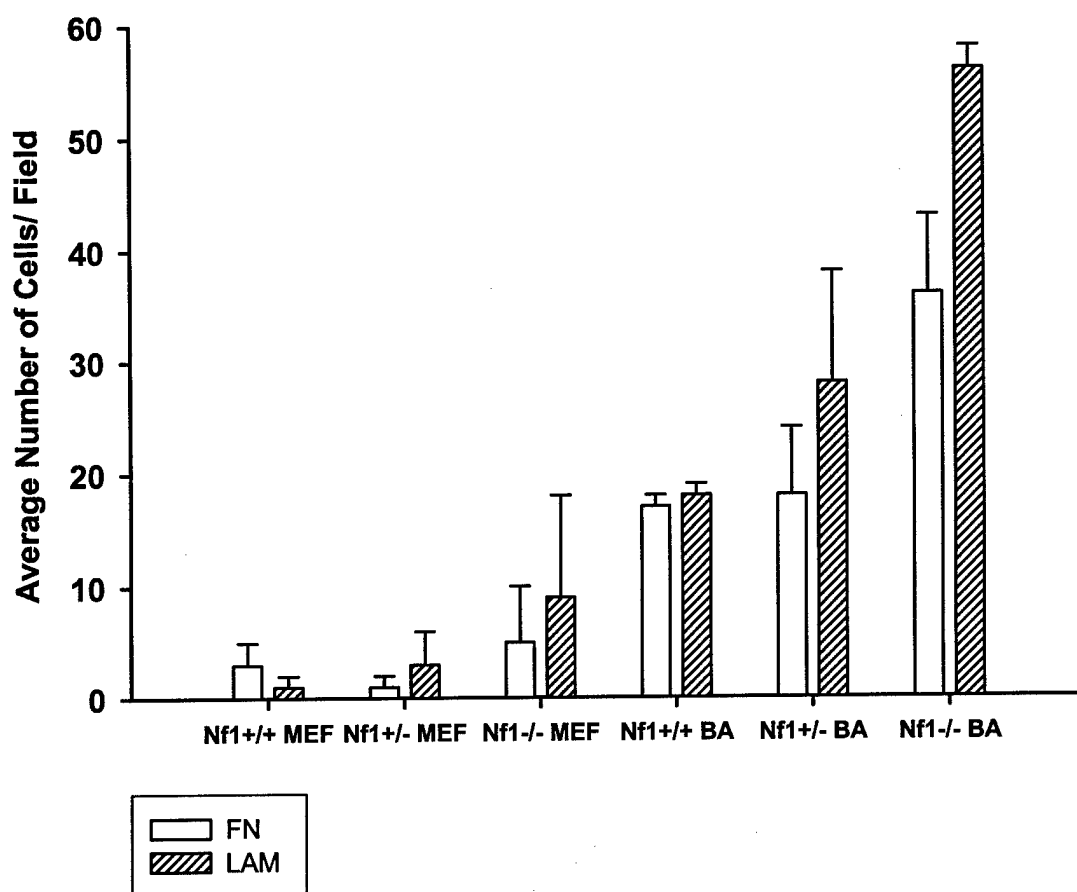


Figure 1B.
Invasiveness of Trigeminal Neural Crest Cells
and Soft Tissue Sarcoma Cells

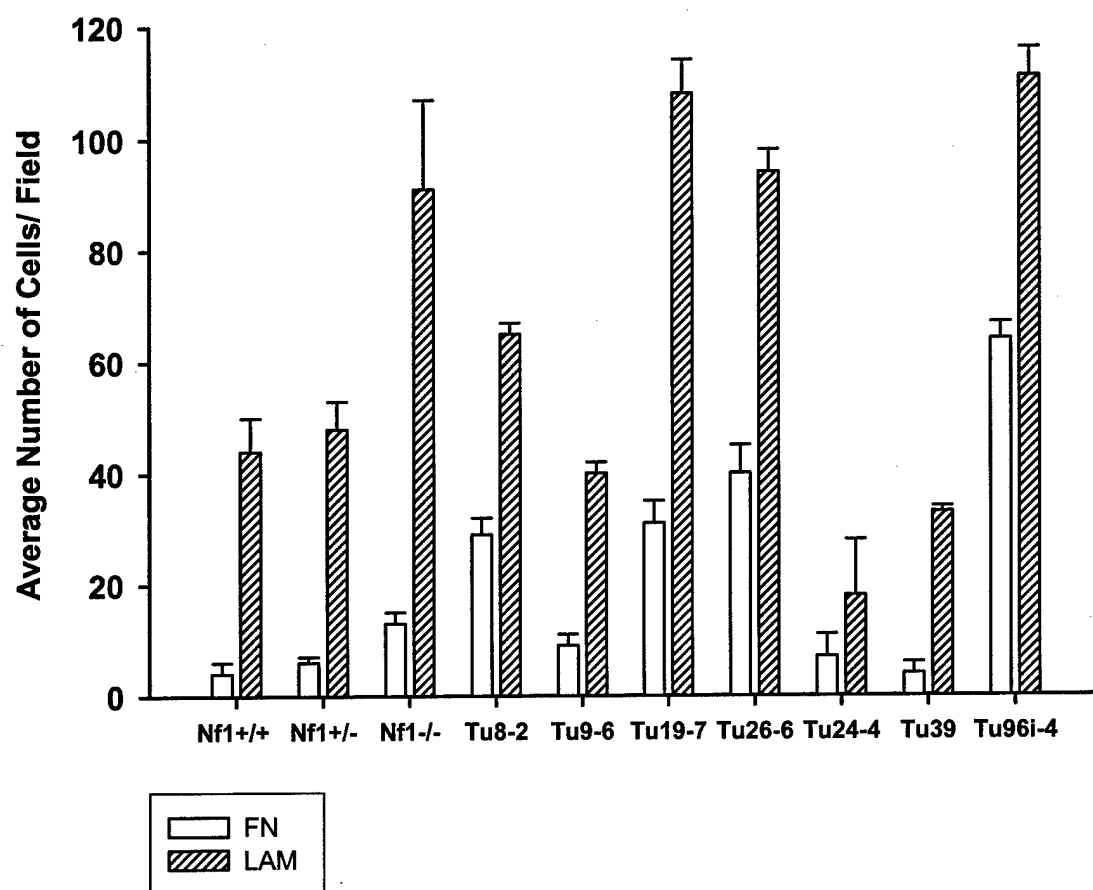


Figure 2A.
Invasiveness of E10 Mandibular Arch
Mesenchymal Cells

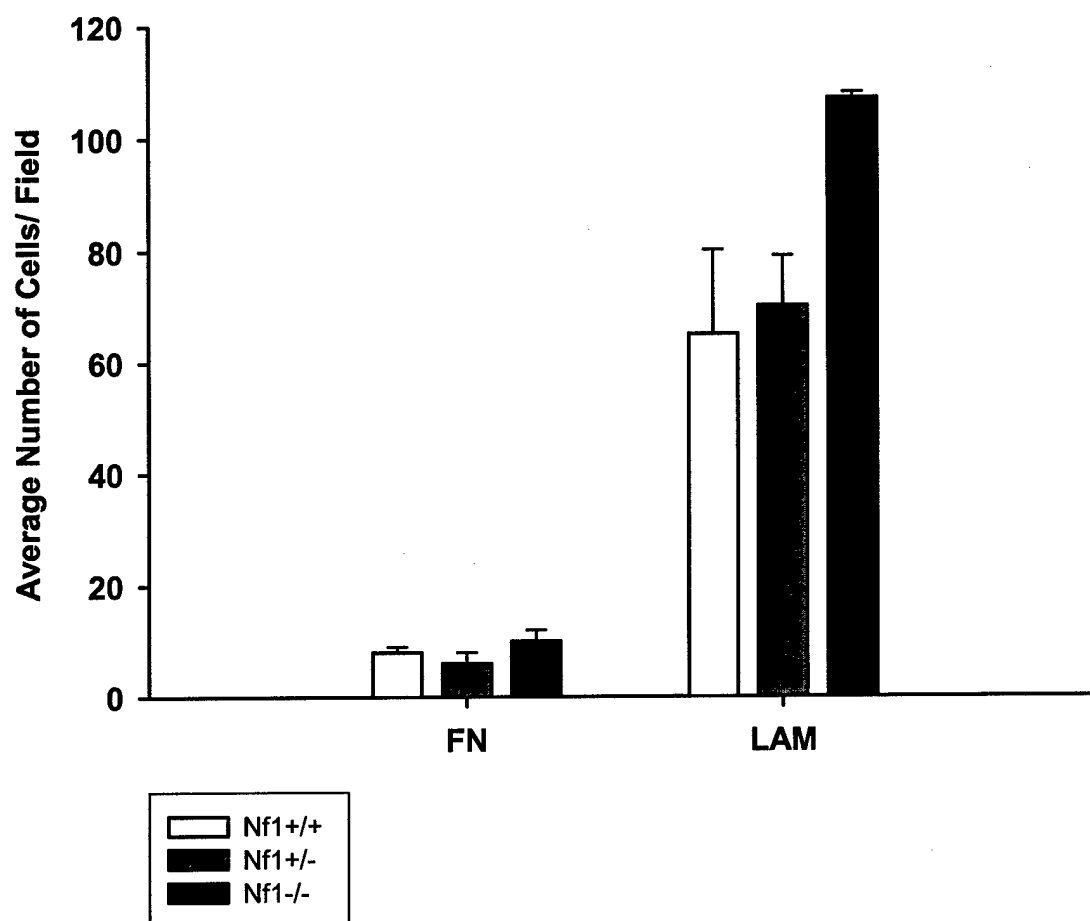


Figure 2B.
Invasiveness of E11.5 Maxillary and
Mandibular Arch Mesenchymal Cells

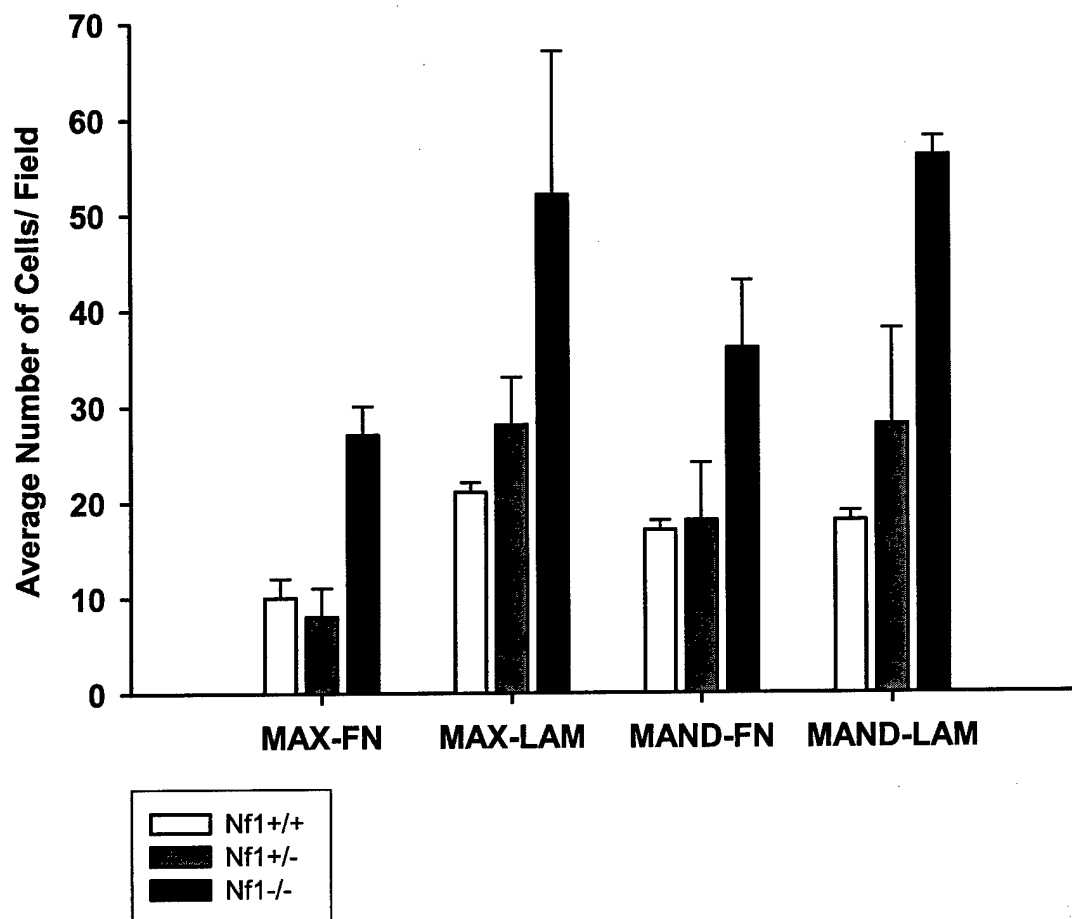


Figure 3A.
Invasiveness of Maxillary Arch Mesenchymal
Cells in Response to FGF2

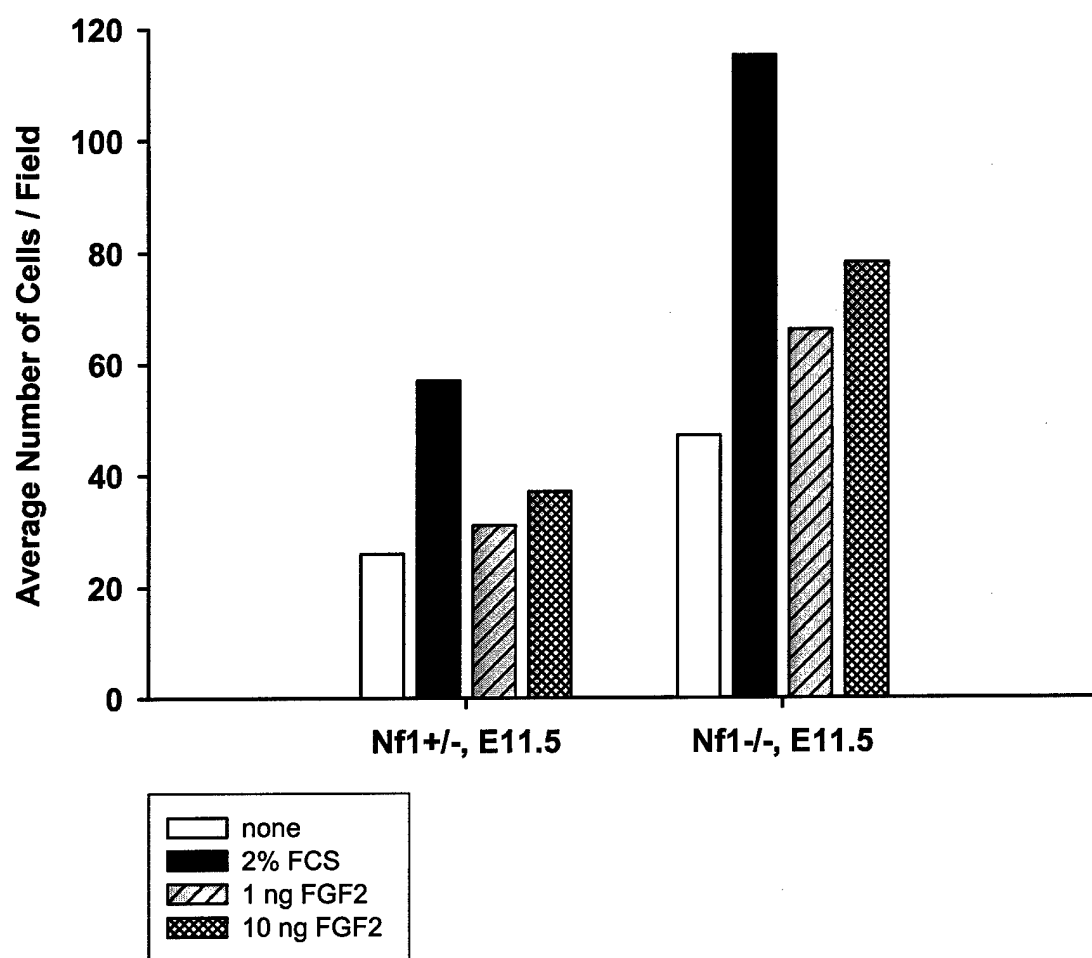


Figure 3B.
Invasiveness of Mandibular Arch Mesenchymal
Cells in Response to FGF2

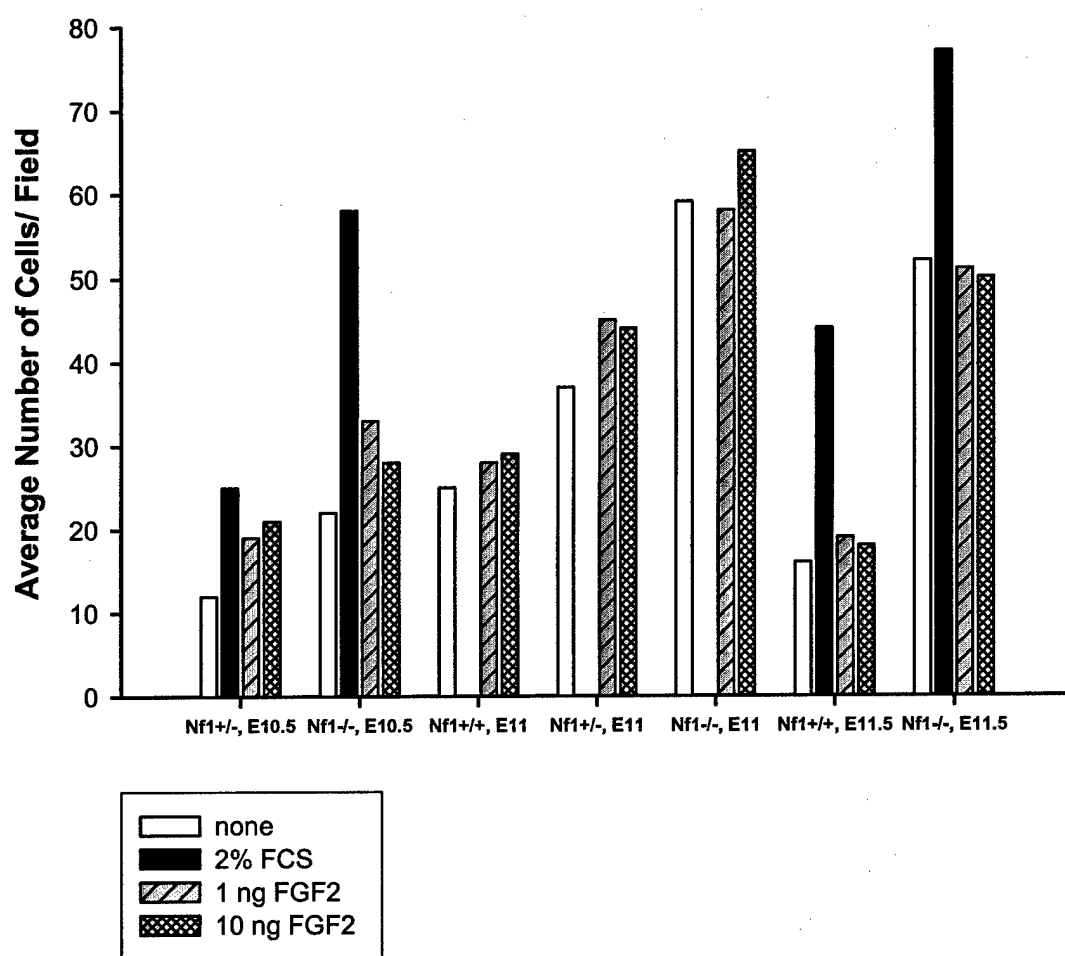


Figure 4A.
Invasiveness of E10.5 Trigeminal
Non-neuronal Cells

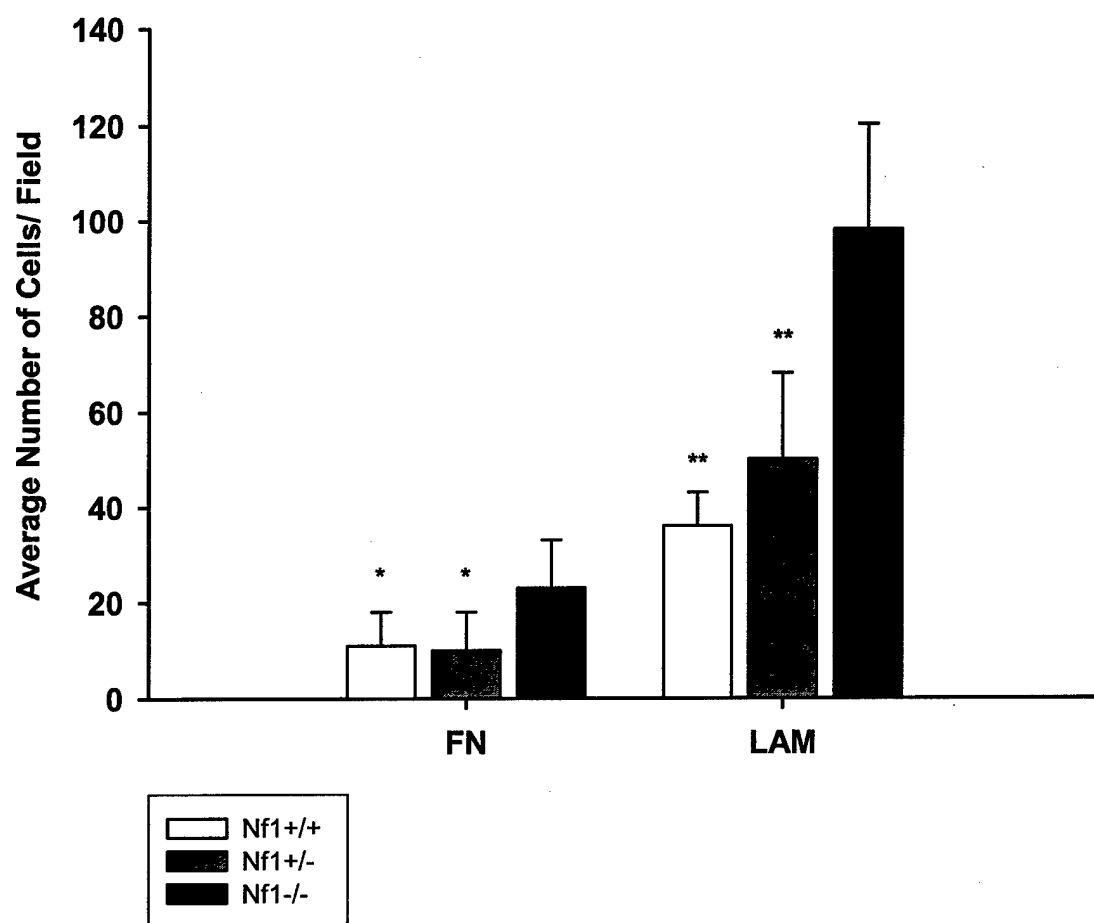


Figure 4B.
Invasiveness of E11.5 Trigeminal
Non-neuronal Cells

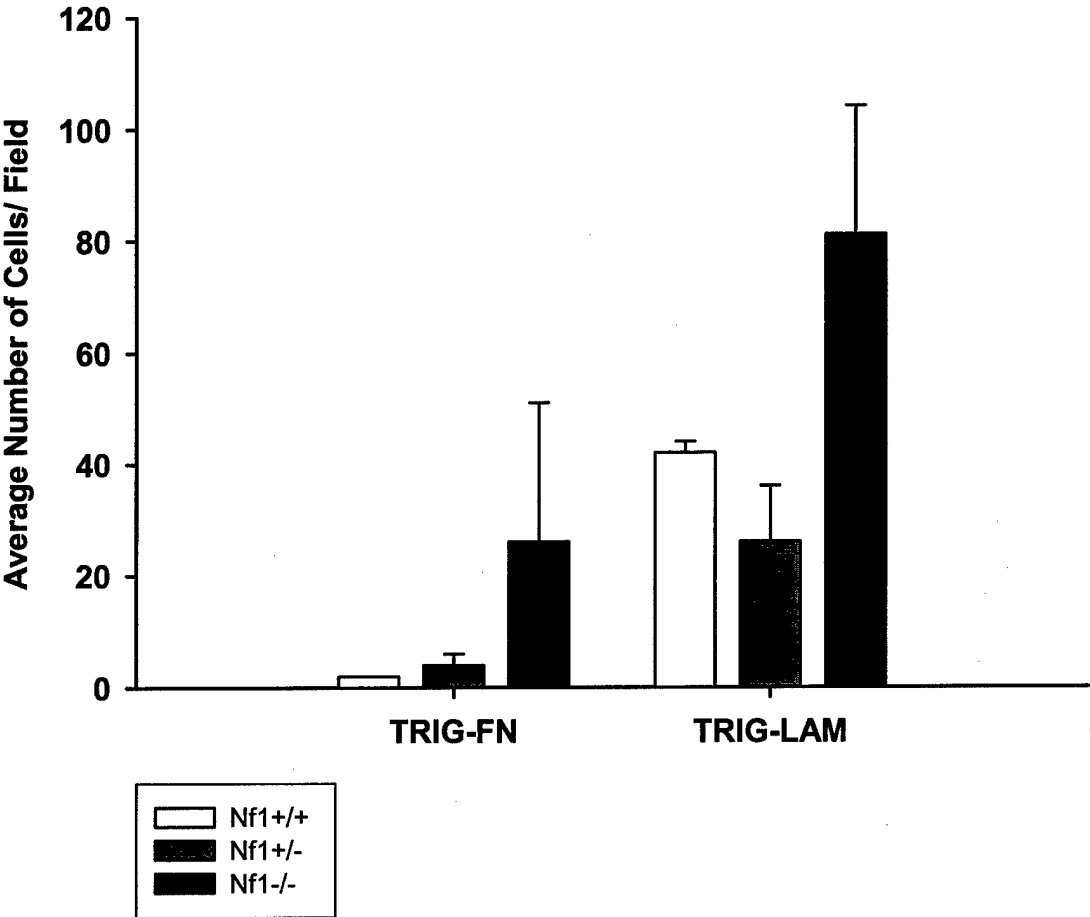


Figure 5.
Invasiveness of E11.5 Trigeminal NCC
Grown in the Presence or Absence of NGF

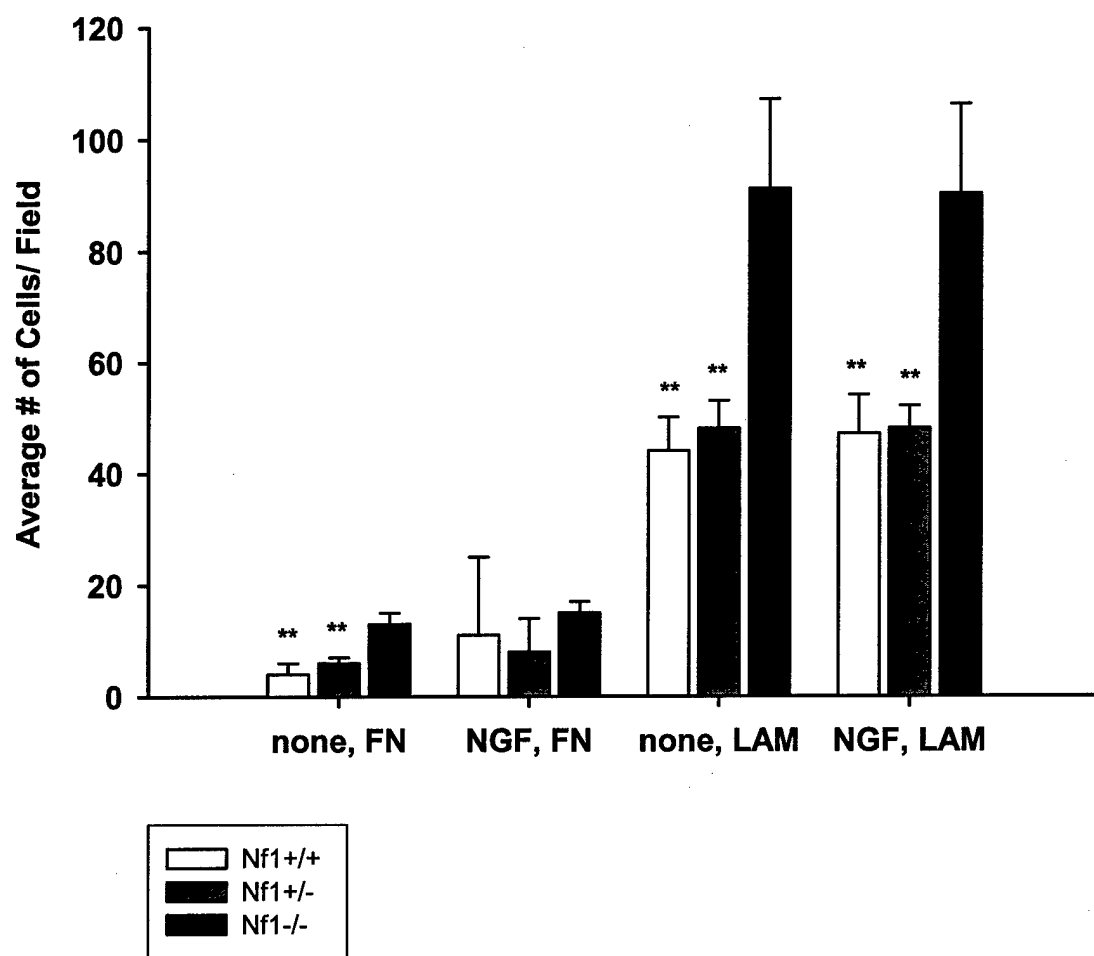


Figure 6.
Effects of PI3 Kinase Inhibitors on Branchial
Arch Mesenchymal Cell Invasiveness

